Replacement of C-Terminal Histidines Uncouples Membrane Insertion and Translocation in Diphtheria Toxin T-Domain

Mykola V. Rodnin,[†] Alexander Kyrychenko,[†] Paul Kienker,[‡] Onkar Sharma,[§] Mauricio Vargas-Uribe,[†] R. John Collier,[§] Alan Finkelstein,[‡] and Alexey S. Ladokhin[†*]

[†]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas; [‡]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York; and [§]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts

ABSTRACT The translocation (T) domain plays a key role in the action of diphtheria toxin and is responsible for transferring the N-terminus-attached catalytic domain across the endosomal membrane into the cytosol in response to acidification. The T-domain undergoes a series of pH-triggered conformational changes that take place in solution and on the membrane interface, and ultimately result in transbilayer insertion and N-terminus translocation. Structure-function studies along this pathway have been hindered because the protein population occupies multiple conformations at the same time. Here we report that replacement of the three C-terminal histidine residues, H322, H323, and H372, in triple-R or triple-Q mutants prevents effective translocation of the N-terminus. Introduction of these mutations in the full-length toxin results in decrease of its potency. In the context of isolated T-domain, these mutations cause loss of characteristic conductance in planar bilayers. Surprisingly, these mutations do not affect general folding in solution, protein interaction with the membranes, insertion of the consensus transmembrane helical hairpin TH8-9, or the ability of the T-domain to destabilize vesicles to cause leakage of fluorescent markers. Thus, the C-terminal histidine residues are critical for the transition from the inserted intermediate state to the open-channel state in the insertion/translocation pathway of the T-domain.

Received for publication 6 July 2011 and in final form 13 October 2011.

*Correspondence: aladokhin@kumc.edu

Alexander Kyrychenko's permanent address is Institute for Chemistry at V. N. Karazin Kharkiv National University, Kharkiv, Ukraine.

Many bacterial toxins enter the cell via the endosomal pathway, which involves bridging of the endosomal membrane in response to acidification as a key step of infection. In diphtheria toxin, this function is carried out by the T-domain that refolds, inserts into the membrane, and translocates the catalytic domain attached to its N-terminus (1,2). The exact molecular mechanism is not well understood, but it is clear that the central issue is a membrane-mediated refolding process, encompassing targeting to the membrane, and a series of interface-directed conformational changes, resulting in multiple charged groups crossing the bilayer and establishing a bilayer-spanning transmembrane (TM) conformation.

Whereas the structure of the soluble T-domain at neutral pH (Fig. 1) has been known for almost two decades (3), little structural information is available for the membrane-associated protein. What is known, is that the insertion/translocation pathway leading toward the final open-channel state (4) contains multiple intermediates (5–9). Almost all studies agree that the most hydrophobic helical pair (TH8-9) inserts across the bilayer in a TM conformation (10–12), while the rest of the structure adopts multiple conformations. Therefore, identifying mutations of titratable residues capable of altering the insertion pathway not only provides insights into the nature of acid-induced conformational switching, but is also potentially useful for future high-resolution studies of key intermediates.

Previously we demonstrated that the pH-dependence of the membrane action of the T-domain is modulated by at least two staggered titration transitions (12). Histidines have been implicated in triggering the pH-dependent conformational changes in the T-domain (7,13), and recently we demonstrated the role of H257 as a major component of the conformational switch leading to destabilization of the folded state in solution (14). The role of the C-terminal cluster of histidines, H322, H323, and H372, however, remains unclear. Based on their location, flanking the consensus insertion domain TH8-9 (Fig. 1 *A*), we hypothesized that their timely protonation is important for the insertion process. To test this hypothesis, we have followed our previous mutagenesis strategy (14) and studied mutants in which all three residues were replaced with either neutral (triple-Q) or positively charged residues (triple-R). Either replacement resulted in similar behavior described below, indicating a particular role of C-terminal histidines in the functioning of the T-domain.

To verify that triple replacement did not cause misfolding of the protein, we measured its secondary structure and thermal denaturation using circular dichroism as described in the Supporting Material and Rodnin et al. (15). We

Editor: Paulo Almeida.

doi: 10.1016/j.bpj.2011.10.018

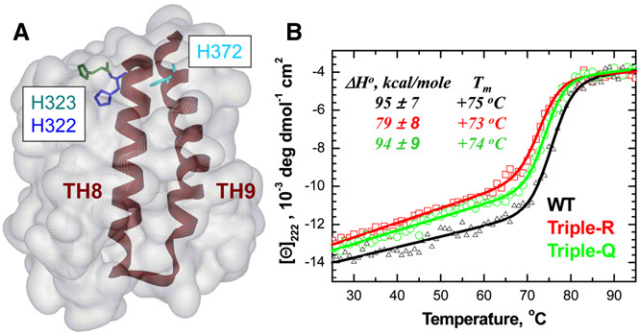

FIGURE 1 (*A*) Crystallographic structure (3) of the WT T-domain of diphtheria toxin (*semitransparent atomic surface*). Consensus membrane insertion domain TH8-9 (*backbone ribbon*). C-terminal histidines H322, H323, and H372 (*colored stick structures*). (*B*) Thermal denaturation of T-domain mutants measured by circular dichroism. (*Symbols*) Ellipticity measurements at 222 nm. (*Lines*) Fits with Eq. S1 (see the Supporting Material). Triple replacements of these residues with either R (*red*) or Q (*green*) do not significantly perturb stability of the protein at pH 8, as judged by changes in enthalpy ΔH^o and transition temperature T_m.

observed only marginal changes in ellipticity of the folded state, unfolding enthalpy ΔH^o, or transition temperature T_m (Fig. 1 *B*). All three proteins also exhibited the same spectral positions of tryptophan fluorescence (not shown). Such behavior differs from that of the previously described H257R mutant, which showed substantial unfolding in solution (14). We conclude that unlike H257, the C-terminal histidines are not involved at the early stages of conformational switching occurring before membrane interaction.

Next, we compared membrane interactions of the triple mutants with those of the wild-type (WT):

First, we determined the kinetics of bilayer insertion by monitoring fluorescence of the environment-sensitive dye NBD selectively attached to a single cysteine residue engineered into the middle of the TH9 helix (Q369C) (Fig. 2 *A*). Previously we demonstrated that such measurements are specific to TM insertion of the consensus domain (12). Our results indicate that insertion of TH8-9 for both mutants is fast and complete at pH 4.5 (Fig. 2 *A*, *solid lines*). The rate of insertion measured at pH 6.0 (Fig. 2 *A*, *dotted lines*) appears to be fastest for the triple-R mutant and slowest for the triple-Q, with WT falling in between. The kinetic variations are consistent with the hypothetical mechanism of formation of the important interfacial intermediate (insertion-competent state), promoted by favorable interaction of protonated histidines with anionic lipids (12). The overall degree of insertion, however, is similar for both mutants to that of the WT at either pH.

Second, we have monitored a general perturbation of the bilayer caused by insertion using a vesicle leakage assay, performed as described before (14). Briefly, large unilamellar vesicles (LUVs) were preloaded with ANTS/DPX dye/quencher mixture and their release was monitored fluorimetrically (the data were normalized to a 100% release level by disrupting the LUVs with Triton-X100). Our results indicate very similar levels of vesicle leakage for WT, triple-R, and triple-Q mutants (Fig. 2 *B*). The requenching analysis (16) indicates a similar all-or-none mechanism of permeation (not shown).

We examined translocation activity of T-domain using proteolytic cleavage of the N-terminal His-tag peptide translocated inside thrombin-loaded vesicles (see Supporting Material). A membrane sample was prepared in which thrombin was placed inside LUVs, and thrombin inhibitor hirudine was added to the outside to ensure that cleavage occurred only for translocated N-terminus. Then, a recombinant T-domain containing a thrombin cleavage site 17-amino-acids downstream from the N-terminus was added and membrane insertion was initiated by lowering the pH. After a 2 h incubation, the sample was solubilized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the appearance of the cleaved T-domain. Our data indicate that translocation-associated cleavage was more efficient in the WT than in the triple mutants, especially triple-R (Fig. 2 *C*).

To confirm that the observed loss of translocating ability in the triple-R and triple-Q T-domain mutants has physiological significance, we introduced the same replacements into the full-length toxin. These measurements were performed using a weakened strain of the toxin carrying the

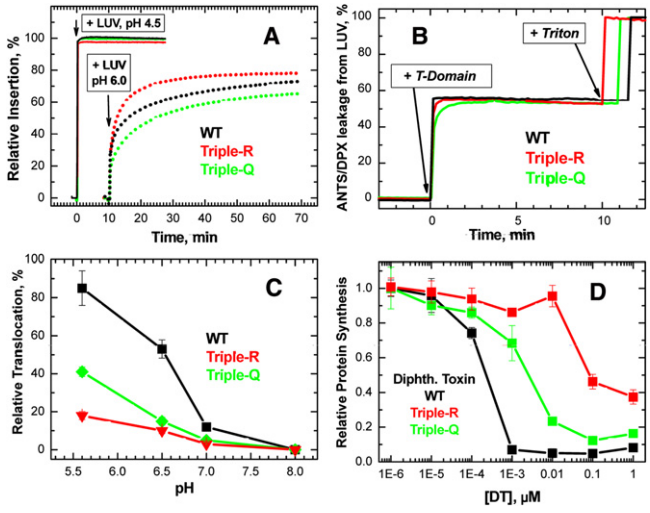

FIGURE 2 Structure-function analysis of the T-domain mutants. (*A*) Insertion of TH8-9 hairpin followed by changes in fluorescence of NBD attached at position 369 as described in Kyrychenko et al. (12). Membrane interaction was initiated by addition of vesicles at times indicated (*arrows*) at pH 4.5 (*solid lines*) or pH 6.0 (*dotted lines*). (*B*) T-domain-induced leakage of vesicle content at pH 4.5. (*C*) Translocation of the *N* terminus measured by proteolytic cleavage as described in the text. (*Error bars*) SD of triplicate measurements. Lipid composition in panels *A*–*C* was 3:1 M:M POPG:POPC. (*D*) Cytotoxicity measurements using the protein synthesis inhibition assay with entire toxin as described in the Supporting Material and Rodnin et al. (14). The loss of functional activity correlates with the loss of translocation, but not of insertion into the bilayer.

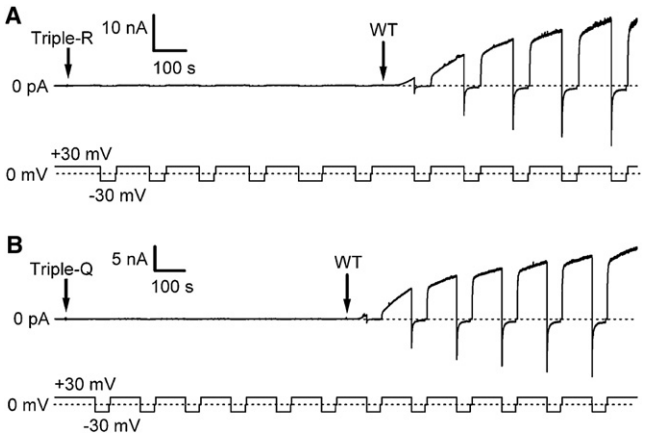

FIGURE 3 Functional activity in planar lipid bilayers of triple-R and triple-Q mutants studied using an electrophysiological assay (1). (*A* and *B*) Record of the transmembrane current (*upper trace*) at ±30 mV voltage (*lower trace*) versus time. For each record, 11 nM of mutant T-domain was added to the *cis* compartment at the start of the record (*first arrow*), followed by addition of 8 nM of WT (*second arrow*). The addition of WT caused a much greater increase in the current than did either mutant. The results indicate a substantial loss of ability to form the open-channel state by the mutants.

E148S mutation in the catalytic domain to reduce the toxic potency (17). Cell death was determined by monitoring the inhibition of protein synthesis. The results indicate that replacements of the C-terminal histidines indeed cause partial loss of function (Fig. 2 *D*), which correlates with the loss of translocation.

Taken together, our results indicate that a cluster of C-terminal histidines, H322, H323, and H372, is important for the functioning of the T-domain and that they are likely to be involved in the late stages of the insertion pathway, perhaps the formation of the so-called open-channel state. To verify this conclusion, we performed conductance measurements using planar lipid bilayers as described previously (1,14), which indeed demonstrate a negligible activity of both mutants compared to that of the WT (Fig. 3). The exact mechanism of involvement of C-terminal histidines on the final stages of insertion/translocation is not yet clear; however, it is reasonable to assume that changes in protonation are required to occur on a very specific step of conformational rearrangement of the T-domain already on the membrane. This type of conformational switching is clearly different from the initial one occurring in solution and involving protonation of H257 (14). We shall continue studies of the molecular mechanism of refolding using various experimental and computational tools, as it is clear that the mutants identified in this study will be instrumental in deciphering the complex insertion pathway of the T-domain.

SUPPORTING MATERIAL

A Methods section with one equation and one figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)01208-2.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants GM-069783 (to A.S.L.), GM-29210 (to A.F.), and AI-022021 (to R.J.C.).

REFERENCES and FOOTNOTES

1. Senzel, L., P. D. Huynh, …, A. Finkelstein. 1998. The diphtheria toxin channel-forming T domain translocates its own NH2-terminal region across planar bilayers. *J. Gen. Physiol.* 112:317–324.
2. Oh, K. J., L. Senzel, …, A. Finkelstein. 1999. Translocation of the catalytic domain of diphtheria toxin across planar phospholipid bilayers by its own T domain. *Proc. Natl. Acad. Sci. USA.* 96:8467–8470.
3. Bennett, M. J., and D. Eisenberg. 1994. Refined structure of monomeric diphtheria toxin at 2.3 Å resolution. *Protein Sci.* 3:1464–1475.
4. Senzel, L., M. Gordon, …, A. Finkelstein. 2000. Topography of diphtheria toxin's T domain in the open channel state. *J. Gen. Physiol.* 115:421–434.
5. Wang, Y., S. E. Malenbaum, …, E. London. 1997. Identification of shallow and deep membrane-penetrating forms of diphtheria toxin T domain that are regulated by protein concentration and bilayer width. *J. Biol. Chem.* 272:25091–25098.
6. Chenal, A., P. Savarin, …, V. Forge. 2002. Membrane protein insertion regulated by bringing electrostatic and hydrophobic interactions into play. A case study with the translocation domain of diphtheria toxin. *J. Biol. Chem.* 277:43425–43432.
7. Ladokhin, A. S., R. Legmann, …, S. H. White. 2004. Reversible refolding of the diphtheria toxin T-domain on lipid membranes. *Biochemistry.* 43:7451–7458.
8. Zhao, G., and E. London. 2005. Behavior of diphtheria toxin T domain containing substitutions that block normal membrane insertion at Pro^{345} and Leu^{307}: control of deep membrane insertion and coupling between deep insertion of hydrophobic subdomains. *Biochemistry.* 44:4488–4498.
9. Montagner, C., A. Perier, …, A. Chenal. 2007. Behavior of the N-terminal helices of the diphtheria toxin T domain during the successive steps of membrane interaction. *Biochemistry.* 46:1878–1887.
10. Oh, K. J., H. Zhan, …, W. L. Hubbell. 1996. Organization of diphtheria toxin T domain in bilayers: a site-directed spin labeling study. *Science.* 273:810–812.
11. Rosconi, M. P., G. Zhao, and E. London. 2004. Analyzing topography of membrane-inserted diphtheria toxin T domain using BODIPY-streptavidin: at low pH, helices 8 and 9 form a transmembrane hairpin but helices 5–7 form stable nonclassical inserted segments on the *cis* side of the bilayer. *Biochemistry.* 43:9127–9139.
12. Kyrychenko, A., Y. O. Posokhov, …, A. S. Ladokhin. 2009. Kinetic intermediate reveals staggered pH-dependent transitions along the membrane insertion pathway of the diphtheria toxin T-domain. *Biochemistry.* 48:7584–7594.
13. Perier, A., A. Chassaing, …, D. Gillet. 2007. Concerted protonation of key histidines triggers membrane interaction of the diphtheria toxin T domain. *J. Biol. Chem.* 282:24239–24245.
14. Rodnin, M. V., A. Kyrychenko, …, A. S. Ladokhin. 2010. Conformational switching of the diphtheria toxin T domain. *J. Mol. Biol.* 402:1–7.
15. Rodnin, M. V., Y. O. Posokhov, …, A. S. Ladokhin. 2008. Interactions of fluorinated surfactants with diphtheria toxin T-domain: testing new media for studies of membrane proteins. *Biophys. J.* 94:4348–4357.
16. Ladokhin, A. S., W. C. Wimley, …, S. H. White. 1997. Mechanism of leakage of contents of membrane vesicles determined by fluorescence requenching. *Methods Enzymol.* 278:474–486.
17. Barbieri, J. T., and R. J. Collier. 1987. Expression of a mutant, full-length form of diphtheria toxin in *Escherichia coli*. *Infect. Immun.* 55:1647–1651.